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Review Article

Chemotherapy targeting cancer stem cells

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Abstract: Conventional chemotherapy is the main treatment for cancer and benefits patients in the form of decreased relapse and metastasis and longer overall survival. However, as the target therapy drugs and delivery systems are not wholly precise, it also results in quite a few side effects, and is less efficient in many cancers due to the spared cancer stem cells, which are considered the reason for chemotherapy resistance, relapse, and metastasis. Conventional chemotherapy limitations and the cancer stem cell hypothesis inspired our search for a novel chemotherapy targeting cancer stem cells. In this review, we summarize cancer stem cell enrichment methods, the search for new efficient drugs, and the delivery of drugs targeting cancer stem cells. We also discuss cancer stem cell hierarchy complexity and the corresponding combination therapy for both cancer stem and non-stem cells. Learning from cancer stem cells may reveal novel strategies for chemotherapy in the future.

Keywords: Side effects, biomarkers, drug delivery system, multifunctional carrier, cancer hierarchy

Introduction: limitations of chemotherapy and corresponding strategies

Other than surgery, radiotherapy, endocrine therapy, and immunotherapy, chemotherapy is the main treatment for cancer. Patients with different cancers derive more survival benefits through chemotherapy not only at the early stage of disease, but also at the late stage. However, quite a few cancers develop drug resistance easily and cause relapse and metastasis. What are the reasons for conventional chemotherapy failure? First, conventional chemotherapy drugs such as paclitaxel mainly target proliferating cancer cells. Such drugs kill the majority of proliferating cancer cells, but cannot do so with dormant cancer cells, which can divide into proliferating cancer cells and cause relapses following chemotherapy [1, 2]. Thus, targeting only proliferating cancer cells is less efficient. Conventional drugs such as cyclophosphamide kill both proliferating and dormant cancer cells [3, 4]. However, multidrug-resistant mechanisms ensure that a number of cancer cells can resist and escape chemotherapy. These dormant or resistant cancer cells are the reason for conventional chemotherapy failure, and are considered cancer stem cells [5-7]. Recently, accumulating studies demonstrated that cancer stem cells, a cancer cell subpopulation with unlimited capacity for self-renewal, differentiation, and tumorigenesis, are the reason for relapse and metastasis [8, 9]. Initially, conventional chemotherapy and radiotherapy kill most cancer cells and shrink tumors immediately, but the spared cancer stem cells eventually result in relapse and metastasis. A new therapy targeting a few cancer stem cells may not shrink the tumor in an obvious manner initially, but may eventually disappear due to the loss of self-renewal and proliferation [10] (**Figure 1**). Other than inhibiting cancer cells, normal tissues are harmed by conventional chemotherapy, which also causes many side effects, such as bone marrow suppression [11], nausea and vomiting [12], neurotoxicity [13, 14], and temporary alopecia [15, 16] due to the targeted drug delivery systems being less precise and the targeting drugs being less efficient. Therefore, enhancing conventional chemotherapy efficacy and reducing its side effects necessitates the search for potential efficient drugs targeting cancer stem cells and designing a novel drug delivery system to trans-

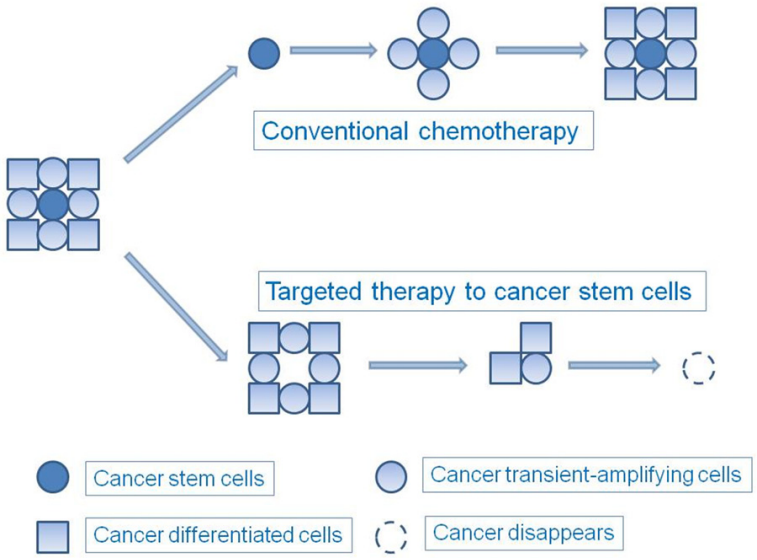

Figure 1. Effects of conventional chemotherapy and targeted therapy. Conventional chemotherapy initially kills most cancer cells and shrinks tumor size immediately, but the spared cancer stem cells eventually result in relapse and metastasis. Targeted therapy of cancer stem cells may not shrink the tumor size in an obvious manner at first, but the tumor may eventually disappear due to the loss of self-renewal and proliferation capacity.

fer such drugs only to cancer sites, and not normal tissues. Herein, we summarize the methods for enriching cancer stem cells, the search for new efficient drugs, and the delivery of targeted therapy drugs. We also discuss cancer stem cell hierarchy complexity and the corresponding target therapy strategies.

Methods of enriching cancer stem cells

Prior to targeted therapy of cancer stem cells, culturing a stable cancer stem cell line for chemotherapy testing is necessary. Ikegaki *et al.* reported the production of several stable neuroblastoma stem cell lines via transient treatment using epigenetic modifiers. A stemness phenotype was maintained by the stable induced cancer stem cells for > 1.5 years in culture with sphere-forming medium [17], providing a new approach for obtaining a phenotypic stable cancer stem cell line. Searching for potential compounds that are preferentially efficacious against cancer stem cells instead of against normal stem cells and differentiated cells is useful. However, whether the suitability of the cancer stem cell line for screening targeted chemotherapy drugs and the suitability of the approach for obtaining other stable cancer stem cell line types remains doubtful. To date, culturing a stable cancer stem cell line is very difficult, as cancer stem cells tend to differentiate rapidly into cancer non-stem cells *in vitro*. However, some agents may be appropriate for cancer stem cell enrichment. The first is phenotypic isolation of cancer cells using specific markers that are mainly expressed in cancer stem cells. Bonnet *et al.* discovered in 1997 that $CD34^+CD38^-$ leukemia cells had much quicker capacity for self-renewal, differentiation, and tumorigenesis than $CD34^-CD38^+$ leukemia cells. Thus, the authors considered $CD34^+CD38^-$ subpopulations the initiating cells of leukemia [18]. Inspired by leukemia stem cell research, other researchers isolated $CD44^+$ $CD24^-$ breast cancer stem cells from patients with breast cancer in 2003 [19]. Subsequently, a number of specific markers were discovered that were related to cancer stem cells [20] (**Table 1**) and which were suitable for enriching cancer stem cells or as potential targets in cancer therapy. A recent study showed that intracellular autofluorescence was an exclusive marker in many epithelial cancer stem cell types. Autofluorescent cancer cells expressed high levels of pluripotency-associated genes, were enriched in sphere culture and during chemotherapy, and had strong capacity for invasion, metastasis, and tumorigenesis [21]. The mechanism of autofluorescence is similar to that of side population cells, a stem-like cell subpopulation [22-24] isolated by Hoechst 33342 efflux, a DNA-binding dye, which depends on the adenosine triphosphate (ATP)-binding cassette sub-family G member 2 [25]. The advantage of this biomarker is that, compared with biomarkers such as CD133, CD44, and aldehyde dehydrogenase (ALDH1), autofluorescent cancer stem cells can be isolated simply by fluorescence-activated cell sorting without requiring a monoclonal antibody or dye, which may affect cancer cells.

The second is enriching cancer stem cells through chemotherapy or radiotherapy. Conventional chemotherapy or radiotherapy are the main treatments for cancer. However, due to resistance, cancer stem cells can

Table 1. Specific cancer stem cell markers

Marker	
A2B5	Glioblastoma [89]
ABCG2	Melanoma [90]
ABCG5	Melanoma [91]
ALDH1	Breast [92, 93], bladder [94], lung [95], colon [96], HNSCC [97], esophageal carcinoma [98]
ANTXR1	Breast [99]
BMI1	Colorectal [10]
CD19	B-precursor ALL [100]
CD26	Colorectal [101]
CD34	AML[102], B-precursor ALL [100]
CD44	Breast [103], colorectal [104], pancreatic [105], ovarian [106], gastric [107, 108], HNSCC [109], AML [110], oral [111]
CD47	AML [112]
CD90	Liver [113]
CD105	Renal [114]
CD110	Colorectal [115]
CD117	Ovarian [106]
CD123	AML [116]
CD133	Brain tumors [34, 117, 118], prostate [119], colon [36, 120], lung [121], melanoma [90], pancreatic [26], ovarian [122], endometrial [123], liver [124]
CD166	Colorectal [104], prostate [125], HNSCC [126]
CD271	Melanoma [127]
CDCP1	Colorectal [115]
CLL1	AML [128]
DDX4	Ovarian [129]
DNAJB8	Renal cell carcinoma [130], colorectal [131]
EGFRvIII	Glioblastoma [132]
EpCAM	Liver [133], colorectal [104]
GD2	Breast [134]
LGR5	Colon [135]
MDR1	Melanoma [136]
OCT4	Osteosarcoma [137]
OV6	Liver [138]
P27	Breast [139]
SOX2	Ovarian [140], cutaneous carcinoma [141]
SSEA1	Glioblastoma [142]
SSEA4	Oral [111]
TIM3	AML [143]

ALL: acute lymphocytic leukemia; AML: acute myeloid leukemia; ALDH: aldehyde dehydrogenase; ABCG: ATP-binding cassette superfamily G member; ANTXR1: anthrax toxin receptor 1; BMI1: B-lymphoma Moloney murine leukemia virus insertion region 1; CDCP1: CUB domain–containing protein 1; CLL1: C-type lectin-like molecule-1; DDX4: DEAD box polypeptide 4; DNAJB8: DnaJ homolog subfamily B member 8; EGFRvIII: epidermal growth factor receptor variant III; EpCAM: epithelial cell adhesion molecule; GD2: glycoprotein D2; LGR5: leucine-rich repeat G protein–coupled receptor 5; HNSCC: head and neck squamous cell carcinoma; MDR1: multi-drug resistance protein 1; OCT4: octamer-binding transcription factor 4; SOX2: sex-determining region Y-box 2; SSEA: stage-specific embryonic antigen; TIM3: T cell immunoglobulin- and mucin domain–containing molecule 3.

escape cytotoxicity and survive chemotherapy and radiotherapy. Therefore, cancer stems cells can be enriched using chemotherapy or radiotherapy. Hermann *et al.* reported that, following 5-day cultivation wih gemcitabine *in vivo*, pancreatic cancer stem cells were enriched up to 47.2% compared to 1.47% in a primary cancer cell line. *In vivo* tumor xenograft experiments showed that, compared to vehicle treatment, the pancreatic cancer stem cells were enriched by > 2 times following 3-week gemcitabine treatment [26]. Dylla *et al.* showed that colon cancer stem cells could survive cyclophosphamide or irinotecan treatment, and in xenogeneic tumors, were enriched. The chemoresistant cancer stem cells expressed more oncogenes, such as *ALDH1A1*, *MYC*, and *MYB* [27]. Bao *et al.* reported that $CD133^+$ glioma stem cells were resistant to radiation by preferentially activating the DNA damage checkpoint response and increasing DNA repair capacity. After radiation, glioma stem cell frequency was increased in both *in vitro* culture and *in vivo* xenograft [28].

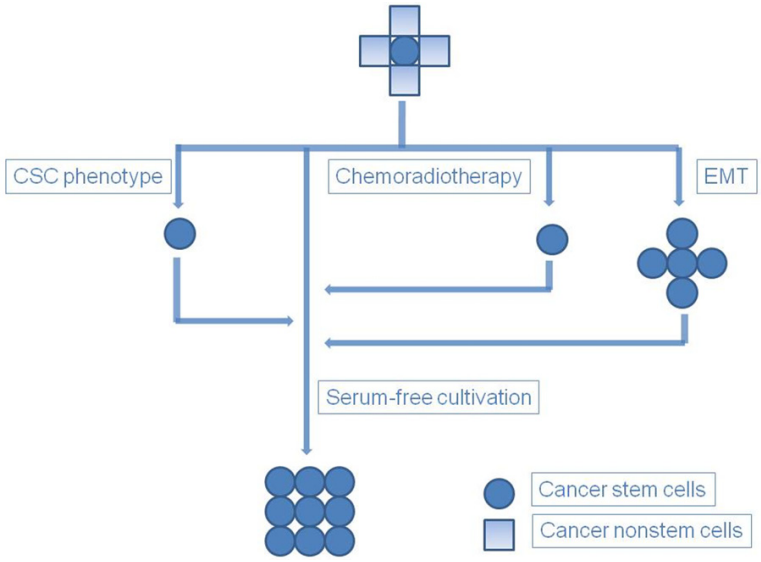

Figure 2. Cancer stem cell enrichment methods. Figure depicts four methods for enriching cancer stem cells (CSC): phenotypic isolation of cancer cells with specific cancer stem cell markers, conventional cytotoxic chemotherapy or radiotherapy, serum-free cultivation, and EMT. The stem-like characteristics of cancer stem cells enriched using other methods require preservation by serum-free cultivation.

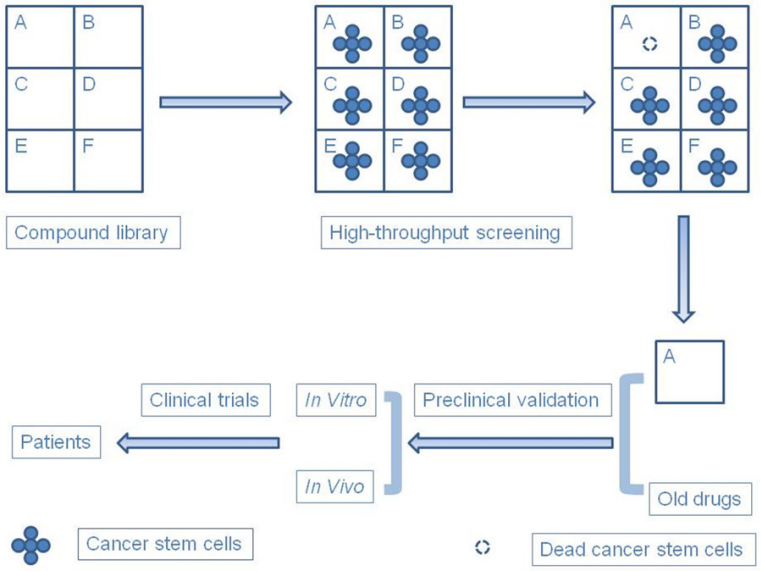

Figure 3. Methods for discovering new efficient drugs. There are two methods for discovering new efficient drugs: High-throughput screening, which is very useful for discovering new drugs among many compounds, and validation of old drugs targeting cancer stem cells.

The third is inducing epithelial to mesenchymal transition (EMT). Mani *et al*. induced immortalized human mammary epithelial cells (HMLEs) into the mesenchymal state through ectopic expression of the Twist or Snail transcription factors, which both induce EMT in epithelial cells. The induced HMLEs formed mammospheres in suspension culture and soft agar colonies *in vitro* effectively, with high and low expression of the surface markers CD44 and CD24, respectively; the authors considered them mammary stem cells or mammary cancer stem cells [29]. The advantage of inducing EMT in cancer stem cells is that there are a large number of induced cancer stem cells and the state is much stabler, which is more suitable for cancer stem cell testing.

The fourth is serum-free cultivation using epidermal or fibroblast growth factor, and other factors. It was first used for enriching neural stem cells [30, 31], and then was used with other normal stem cells such as mammary stem cells [32, 33]. Due to the lack of specific cancer stem cell markers, it was used in the last decade to enrich cancer stem cells, such as that from brain [34], breast [35], colon [36], pancreatic [37], and prostate cancer [38]. The benefit of serum-free cultivation is that it preserves the state of stemness. This method preserves the stem-like characteristics of cancer stem cells enriched by other methods.

These four methods can be used to enrich cancer stem cells (**Figure 2**). Their common drawback is that the enriched cancer cells are not pure cancer stem cells. Therefore, using two or more methods to enrich cancer stem cells is more suitable.

Methods of searching for new efficient drugs

How do we search for new efficient drugs targeting cancer stem cells? A high-throughput screening platform may be one option (**Figure 3**). Gupta and colleagues screened 16000 compounds, eventually selecting salinomycin,

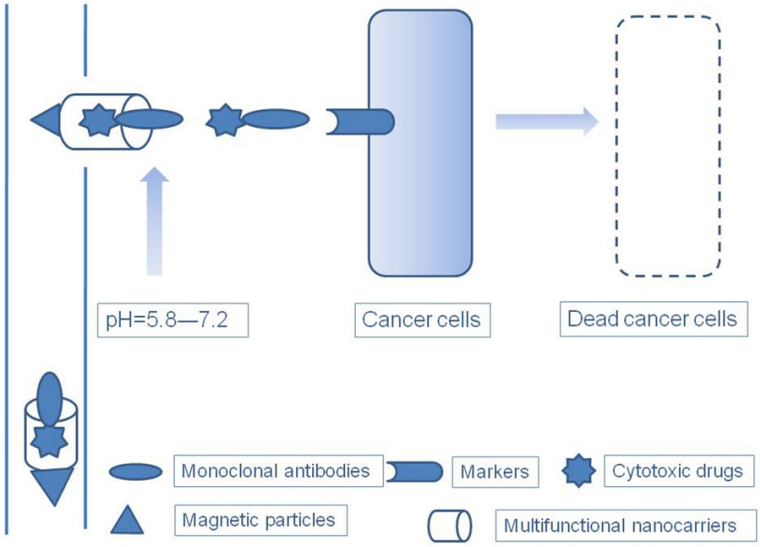

Figure 4. An ideal drug and drug delivery system. The ideal drug and drug delivery system should combine passive targeting aspects, e.g., enhanced permeability and retention (EPR) effect of the tumor; pH-, light-, and thermosensitive; and magnetic properties, with active targeting using monoclonal antibodies specific to cancer. Multifunctional nanocarriers are ideal carriers for chemotherapy drugs, where they adopt the tumor EPR effect, conjugate with one or more pH-, light-, and thermosensitive and magnetic particles, and load cytotoxic drugs and monoclonal antibodies targeting cancer.

which inhibits breast cancer stem cells 100-fold more effectively than paclitaxel, the main drug for breast cancer chemotherapy [39], which proved to be a breakthrough for screening drugs that target cancer stem cells. Many studies followed these findings [40-43]. However, some researchers were critical of the fact that salinomycin is very toxic in normal cells and causes lethal side effects, and may be not suitable for chemotherapy *in vivo* [44].

Another option is validating old drugs that inhibit cancer stem cells efficiently (**Figure 4**), such as metformin, which is used for diabetes. Cancer risk is reduced in patients with diabetes who receive metformin [45-49]. Metformin inhibits cancer stem cell sphere-forming *in vitro* and xenografts *in vivo*, and sensitizes many cancers, such as breast [50-53], pancreatic [54, 55], and colon cancer [56], and esophageal carcinoma [57] and glioma [58] to radiotherapy and chemotherapy. Phenformin, a related biguanide, inhibits lung cancer stem-like cell growth and invasive capacity *in vitro* [59], and affects the metabolic state of breast cancer stem cells [51]. In addition to metformin and phenformin, the anti-alcoholism drug disulfiram is markedly cytotoxic in cancer stem-like cells of breast cancer [60, 61], hepatocellular carcinoma [62], and glioblastoma [63, 64]. It inhibits self-renewal, induces apoptosis, and reverses drug resistance through mechanisms such as inducing reactive oxygen species, inhibiting the ALDH and nuclear factor-κB (NF-κB) pathways, downregulating glypican-3, inhibiting chymotrypsin-like proteasomal activity, and inactivating the ubiquitin-proteasome pathway. The antipsychotic drug thioridazine selectively targets leukemia stem cells via the dopamine receptors, but without being cytotoxic to normal blood stem cells [65]. Its anti-cancer potential was also reported in breast and gastric carcinoma [66, 67]. Some dopamine analogues also inhibit glioblastoma stem cells efficaciously [68]. In addition to these drugs, more drugs targeting cancer stem cells need to be discovered and validated in clinical trials before clinical usage.

Methods of delivering cancer-targeting drugs

Delivering anti-cancer drugs specific to cancer tissues and sustaining a stable high drug concentration improve anti-cancer efficacy and reduce the side effects in normal tissues. Some characteristics of cancer may be used to realize this. First, due to form and architecture abnormality of the newly formed blood vessels, the vascular endothelial cell interstitial space in cancer tissues is much looser than that in normal tissues. This allows anti-cancer drugs to infiltrate into the cancer tissues easily if the drug molecule is the same size as that of the gap between normal tissues and cancer tissues. In addition, the lack of effective lymphatic drainage ensures that the drug is much more easily retained in cancer tissues than in normal tissues. This is termed the enhanced permeability and retention effect, which is widely used in anti-cancer drugs modified with liposomes, nanomaterials, or high–molecular weight polymers [69]. Moreover, the pH values of normal tissues and cancer tissues differ. Due to the

stronger glycolysis, cancer tissues generate more lactic acid, therefore the cancer microenvironment pH is about 5.8–7.2 (median, 7.0); under normal conditions, the pH is generally around 7.4 [70, 71]. A pH-sensitive drug is designed to release slowly under normal conditions, but the stability of a complex drug decreases under the pH conditions of cancer tissue, such that it is released quickly mainly in cancer tissue and kills cancer cells specifically [72]. In addition to the specific cancer tissue physical and chemical properties, a complex drug can have light-sensitive [73], thermosensitive [74], and magnetic properties [75], and then illumination, heat, and magnetization external to the tumor location draw the drug specifically into the cancer tissues. However, special equipment is needed for each treatment session, rendering it less convenient.

The above methods for anti-cancer drug delivery are considered passive targeting. Conversely, active targeting delivers anti-cancer drugs by conjugating the complex drugs with monoclonal antibodies specific to the target cancer tissues. In comparison to normal cells, cancer cells have abnormal molecular expression, cell signaling pathways, and microenvironments, which are potential targets for guiding anti-cancer drugs with specific monoclonal antibodies [9, 76, 77]. For example, herceptin, or trastuzumab, a monoclonal antibody of human epidermal growth factor receptor 2 (HER2), which is overexpressed in some breast cancers, is widely used to treat HER2-positive breast cancer. Choi *et al.* reported that a herceptin-conjugated, doxorubicin-loaded multifunctional nanocarrier led to much higher cellular uptake and stronger cytotoxicity in HER2 overexpression breast cancer *in vitro* and shrank tumors significantly *in vivo*, compared to that not conjugated to herceptin [78]. Several studies have also shown that different herceptin-conjugated cytotoxic drugs loaded to multifunctional carriers improved therapy efficacy in HER2-positive breast cancer [79, 80] and pancreatic cancer [81]. This active targeting method tends to inhibit cancer with high efficacy, and a greater number of specific cancer cell or cancer environment targets need to be discovered for potential active targeting drug design.

Recently, nanomedicine has come to the fore in cancer drug design and delivery [82, 83]. Multifunctional nanocarriers combine passive and active targeting methods, which the ideal anti-cancer drugs should have, to enhance their efficacy and to reduce side effects (**Figure 4**). Chiang *et al.* designed a multifunctional nanocarrier with passive targeting pH-sensitive and magnetic particles, the active targeting herceptin, and the cytotoxic drugs doxorubicin (hydrophilic) and paclitaxel (hydrophobic). The complex compound, containing three agents, enhanced anti-cancer efficacy more efficiently than a nanocarrier with only one or two agents [79]. Its advantage is that it is more convenient, and patients with cancer would prefer one complex drug rather than the combination therapy in the present clinical treatment, which involves several drugs.

Learning from cancer stem cell hierarchy complexity

In addition to cancer stem cells, tumors contain the bulk of differentiated cancer cells. Currently, the relationship between cancer stem cells and differentiated cancer cells is not well known. According to the cancer stem cell hypothesis, cancer stem cells head the hierarchy, and can differentiate into transient amplifying and differentiated cancer cells [20, 84]. Conversely, transient amplifying and differentiated cancer cells cannot dedifferentiate into cancer stem cells. The hierarchy is similar to that of normal stem cells; however, recent studies have disputed this. Takahashi and Yamanaka reported that pluripotent stem cells were generated directly from fibroblast cultures following the addition of four genes, namely *OCT3/4*, *SOX2*, c-*MYC*, and *KLF4* [85]. On some occasions, differentiated cancer cells can also convert to cancer stem cells. Chaffer *et al.* reported that a subset of oncogene-transformed basal-like mammary epithelial cells spontaneously dedifferentiated into cancer stem-like cells with tumorigenesis capacity [86]. Schwitalla *et al*. reported a similar observation, where NF-κB– and β-catenin–transformed differentiated vellus cells dedifferentiated into stem-like cells and could form spheroids *in vitro* and cancer *in vivo* [87]. The cancer stem cell and differentiated cancer cell interconversion indicates a more complex cancer stem cell hierarchy. Therefore, therapy that only targets cancer stem cells may be less effective than initially presumed, and combination therapy targeting

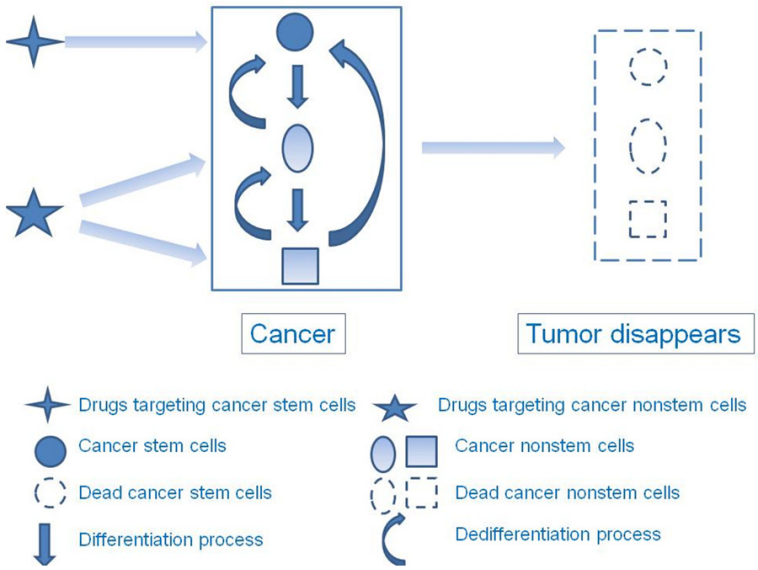

Figure 5. Complexity of cancer stem cell hierarchy and corresponding combination therapy. Cancer stem cells are usually at the top of the cancer hierarchy and can differentiate into transient amplifying and differentiated cancer cells. Cancer non-stem cells can also regain self-renewal and proliferation capacity through dedifferentiation under certain conditions. The complexity of cancer stem cell hierarchy indicates that the appropriate treatment strategy is combination therapy simultaneously targeting cancer stem and non-stem cells.

cancer stem cells and differentiated cancer cells may be more effective. Zhang *et al.* reported that paclitaxel, the main drug in breast cancer chemotherapy, combined with salinomycin, a high-throughput screening–validated drug that targets breast cancer stem cells [39], was more efficient for eradicating breast cancer and cancer stem cells compared to treatment with only one drug. The effect was improved when paclitaxel was modified with polyethylene glycol-block-polycaprolactone (PEG-b-PCL) polymeric micelles and octreotide, which targets somatostatin receptors overexpressed in breast cancer, and when salinomycin was modified with PEG-b-PCL polymeric micelles [88]. Ke *et al.* reported that using polymeric micelles for co-delivering thioridazine, which targets breast cancer stem cells effectively, and doxorubicin, a conventional cytotoxic drug used for treating breast cancer, was more effective for inhibiting both cancer stem and non-stem cells compared to delivering only one drug [66]. In conclusion, the complexity of cancer stem cell hierarchy teaches us that combination therapy should simultaneously target the bulk of cancer non-stem cells and some cancer stem cells (**Figure 5**).

Conclusion

One solution to enhancing conventional chemotherapy efficacy and reducing its side effects is modifying the drug delivery system and discovering more efficient target drugs. An ideal drug delivery system must deliver drugs only to cancer sites and sustain high, stable drug concentrations. An ideal complex drug should target all cancer cells, and not normal cells; a multifunctional nanocarrier can meet both demands. Given cancer stem cell hierarchy complexity, a strategy involving combination therapy should be used to target both the bulk of differentiated cancer cells and the minority of cancer stem cells together. The bulk of stable cancer stem cells are indispensible for testing drug effects in the search for more efficient drugs that target cancer stem cells. The four cancer stem cell enrichment methods described earlier are phenotypic isolation of cancer cells with specific cancer stem cell markers, conventional cytotoxic chemotherapy or radiotherapy, suspension cultivation, and EMT. A high-throughput screening platform may be a better choice for screening efficient target drugs. Learning from cancer stem cells, combining new drug delivery systems, and new target drugs may reveal novel strategies for chemotherapy in the future.

Disclosure of conflict of interest

None.

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